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(57) Abstract

The present invention relates to a method of amplifying a nucleic acid sequence present in a first strand of a double stranded nucleic acid molecule wherein said molecule incorporates an unmodified recognition site for a restriction enzyme capable of cutting the first strand at the 5' end of the sequence therein to be amplified and leaving the 3'–end region of the second strand projecting beyond the cut site in the first strand. The method further comprises treating said molecule with said enzyme in the presence of a strand displacing polymerase and unmodified nucleotides for incorporation in an extending nucleic acid strand such that there is or becomes hybridised to said 3'–end region of the second strand a primer sequence complementary thereto whereby said primer sequence is extended in the 5' to 3' direction using the second strand as a template to regenerate the restriction endonuclease cut site and displace the sequence to be amplified.

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AMPLIFICATION OF NUCLEIC ACIDS

The present invention relates to the amplification of nucleic acids, i.e. procedures for producing copies of nucleic acid sequences.

As used herein the term "nucleic acid" includes protein nucleic acid (PNA) (i.e. nucleic acids in which the bases are linked by a polypeptide backbone) as well as nucleic acids (e.g. DNA and RNA) having a sugar phosphate backbone.

Various nucleic acid amplification techniques are already known, e.g. the Polymerase Chain Reaction (PCR). However many of these techniques (including PCR) suffer from the disadvantage that various cycles of heating and cooling are required for each amplification reaction. Thus, in a typical amplification reaction, the sequence (in single stranded form) to be amplified is treated with an oligonucleotide capable of hybridising to the sequence at a particular location thereof, the treatment being effected at a temperature (and under other conditions, e.g. buffers etc.) at which the hybridisation will occur. In the next step (which may or may not be effected at the same temperature) a polymerase enzyme is used to extend the oligonucleotide primer (using the original sequence as a template) to produce a strand which is complementary to the original strand and which is hybridised thereto. Subsequently the reaction mixture must be heated to denature the complementary strands and then cooled so that the above described procedure (i.e. primer hybridisation, extension, denaturing) may be repeated.

An alternative amplification procedure known as Strand Displacement Amplification has previously been proposed. This procedure may be effected isothermally but does require the construction of a double stranded nucleic acid molecule incorporating hemi-modified restriction site, more particularly a site modified (in one strand) by the incorporation of a thiolyated adenine. The SDA reaction must be conducted in the presence of a chemically modified base to ensure

regeneration of the hemi-modified restriction site. This modified base does however become incorporated in the copy strands produced in the reaction and this is a restriction imposed on the procedure.

According to the present invention there is provided a method of amplifying a nucleic acid sequence present in a first strand of a double stranded nucleic acid molecule comprised of complementary first and second strands wherein

said molecule incorporates an unmodified recognition site for a restriction enzyme capable of cutting the first strand at the 5' end of the sequence therein to be amplified and leaving the 3'-end region of the second strand projecting beyond the cut site in the first strand, and said method comprises

treating said nucleic acid molecule with said enzyme in the presence of a strand displacing polymerase and unmodified nucleotides for incorporation in an extending nucleic acid strand under conditions such that there is or becomes hybridised to said 3'-end region of the second strand a primer sequence complementary thereto whereby said primer sequence is extended in the 5' to 3' direction using the second strand as a template to re-generate the restriction endonuclease cut site and displace the sequence to be amplified.

By unmodified recognition site we mean that the site consists of unmodified A, G, T and/or C bases.

It will be appreciated that the steps of cutting and extension may be repeated as often as necessary to generate the desired amplification.

An important feature of the invention is that the restriction enzyme is capable of providing a 3'-end region of the second strand which projects beyond the cut site in the first strand. The nucleic acid molecule and/or the nature of the restriction enzyme

may be such that only the first strand is cut (i.e. nicked). Alternatively both strands may be cut. In either case, the cut in the first strand generates a fragment (a 3'-upstream fragment) on the 3' side of the cut in that strand. This fragment may, in certain cases, act as said primer sequence (provided that it remains hybridised to the 3'-end region of the second strand) Alternatively, depending on the length of the fragment and/or the reaction conditions, the fragment may be cleaved from the end region of the second strand to generate a 3'-overhang. It is therefore usually preferred that an oligonucleotide primer (also referred to herein as the FP primer) capable of hybridising to the 3'-end region is additionally incorporated in the reaction to improve the probability of there being a primer sequence hybridised to the 3'-end region of the second strand for effecting the extension/displacement reaction. It will be appreciated that the FP primer incorporates all or part of the overhanging sequence produced by the enzyme digestion outlined above.

The manner in which amplification proceeds to effect amplification is described in more detail below but, in brief, the primer sequence is extended in the 5' to 3' direction to displace the sequence to be amplified whilst creating a further copy of that sequence (hybridised to the template strand) and regenerating the restriction site. The processes of cutting the double stranded molecule and extension/displacement are repeated to provide for increasing quantities (i.e. amplification) of the sequence to be amplified.

It is preferred that the nucleic acid molecule incorporates two restriction sites of the type described, one each side of the sequence to be amplified. These restriction sites are ideally the same as each other (so that only one restriction enzyme is required) but may be different. The provision of two restriction sites as described allows for extension/displacement reactions to proceed in opposite directions from either end of the molecule. By providing an excess of FP primers in the reactant mix, these primers may hybridise to the 3'-overhangs, produced by the digestion with the restriction enzyme, allowing production of double stranded molecules incorporating a

single restriction site. These double stranded molecules participate in further amplification reactions as described more fully below. As described below, such further reactions produce nucleic acid strands which are not able to bind to the FP primers. In an advantageous development of the invention, the reactant mix includes at least one further type of primer (referred to herein as ISOS primers) incorporating the FP sequence and being capable of hybridising to the nucleic acid strands which are themselves not able to bind to the FP primers per se. The ISOS primers result in generation of further double stranded molecules incorporating a restriction site, such molecules being able to participate in amplification reactions. This channelling of otherwise non-hybridisable single strands back into the amplification process leads to an exponential accumulation of product. Where both ISO-S and FP primers are used, the former will generally be employed at a much lower concentration than the latter, generally at least 10 fold less ISO-S primer than FP primer. The ISO-S primers generally are used at a concentration of between 1 fmol/μl to 50 pmol/μl and the FP primers are generally used at a concentration of between 10 fmol/μl to 500 pmol/μl. It should be noted that, under conditions in which the cleavage products of restriction enzyme digestion do not become separated prior to the action of the DNA polymerase, the exponential amplification reaction may be performed by the ISOS primers in the absence of FP primers.

The method of the invention may be a solution phase reaction, and must occur under such conditions (of salt concentration, pH, nucleotide concentration etc.) that both the restriction digestion and polymerase extension reactions can occur, though not necessarily simultaneously. Preferred conditions for the method of the invention to be carried out are in a New England Thermopolymerase buffer at pH 8.8, in the presence of 10-20 mM magnesium ions, and a nucleotide final concentation of 0.1 - 1.0 mM.

Theoretically, the target nucleic acid for the amplification may be present in very small amounts, and hence the amplification will find utility in such areas as

clinical diagnostics, to detect either the presence or absence of a sequence or, at a more discriminatory level the occurrence of variations of an underlying sequence. Both DNA and RNA might be used as the target for amplification, the later requiring initial reverse transcription to a DNA intermediate.

The restriction enzyme may for example be *Tsp*RI but any other enzyme producing the required 3'-end region in the second strand may be used.

The strand displacing polymerase may for example be 9°N polymerase (ex-New England Biolabs), Klenow (exo-) polymerase, Bst polymerase, Vent (exo-) polymerase, or Deep Vent (exo-) polymerase.

The temperature(s) at which the reaction is effected is/are dependent upon the combination of restriction endonuclease and displacing polymerase used in the reaction. Under certain conditions, the restriction endonuclease will effect cutting at the temperature at which the displacing polymerase will effect a copying reaction. Under such conditions, the reaction of the invention may be effected isothermally. It is however also within the scope of the invention that different temperatures are required for cutting and copying so that a two-step thermal cycling reaction may be envisaged. Thermal cycling in this case is not that cycling which is required to perform PCR. In PCR, a strand separation step is an absolute requirement of each cycle of the technique and is usually performed by heating the sample to 95-98°C. No such cyclic strand separation is required in the method of the invention where cycling is used to allow primer annealing or to move between the temperature optima of the enzymes used.

The invention will be further described by way of example only with reference to the accompanying drawings, in which

Fig. 1 illustrates the recognition site for the restriction enzyme *Tsp*RI;

Fig. 2 schematically illustrates a DNA molecule for amplification in accordance with the method of the invention;

Fig. 3 schematically illustrates one embodiment of the method;

Fig. 4 schematically illustrates a further embodiment of the method;

Figs. 5-11 illustrate the results of the Examples.

Referring to the drawings, Fig. 1 illustrates the restriction site of the enzyme *Tsp*RI. This recognition sequence comprises five base pairs as shown and the enzyme cuts two bases either side of the recognition sequence to produce a 9-base 3' overhang.

Reference is now made to Fig. 2 which illustrates a double stranded target DNA molecule 1 containing an internal sequence X (flanked by sequences Y and Y') to be amplified by the procedure described more fully below with reference to Fig. 3. With reference to these figures, the following convention will be used when referring to the composition of fragments. Sequences which are complementary are denoted by the same letters (or sequence of letters) but with one of the two complementary sequences additionally being denoted by the "prime" suffix ('). Thus X' is the complementary sequence to X.

When a molecule is composed of more than one segment it will be annotated from the 5' end of the molecule (thus the notation YXY' would be used to denote the upper strand of the molecule in Fig. 2). When a double stranded molecule is referred to the upper and lower sequences are separated by a slash (/) with both annotated from their 5' ends. Thus the entire molecule shown in Fig. 2 can be denoted YXY'/YX'Y'.

The molecule shown in Fig. 2 has a *Tsp*RI restriction site towards each end thereof flanking the sequence X/X' with each site being spaced from its respective end of the molecule by a short (8 bp) sequence to distance the restriction site away from the 5' end of the molecule. These 8 bp sequences, which are identical at each end of the molecule, have been included because certain restriction enzymes are unable to

cleave near the ends of DNA. It has not been reported whether *Tsp*RI behaves in this fashion so that this sequence may or may not be necessary. Likewise, the optimum length of this sequence has not been reported and so this spacer may contain more or less than 8 bases.

It will be appreciated that the 17 base sequences at the 5' ends of the two strands (A and B) of the molecule 1 are identical and are referenced in Fig. 2 as Y and Y'.

In order to effect the amplification reaction, the molecule 1 is treated with the enzyme *Tsp*RI in the presence of a strand displacing polymerase, a primer FP1 having the aforementioned sequence Y, and the four nucleotides in a buffer such as conventionally used in amplification reactions.

A manner in which the reaction may proceed is illustrated in Fig. 3 in which the dashed vertical line is included purely to illustrate the relationship between the various steps of the process.

Initially, the molecule 1 is cleaved at both ends by *Tsp*RI so as to produce a "cleaved" molecule having 3' overhangs (each 9 bases) as illustrated as the result of step (a) in Fig. 3. In the next step, the primer FP1 (shown in bold for the purposes of clarity) hybridises to the "cleaved" molecule to produce the construct illustrated as the product of step (b) of Fig. 3. It should at this point be appreciated that exactly the same construct would be obtained if the short cleavage fragments of sequence Y nicked by the *Tsp*RI did not become denatured or if such denaturation did occur they became rehybridised.

In the next step, the strand displacing polymerase begins to extend each primer FP1 so that (with reference to the primer FP1 shown at the left hand end of Fig. 3) the primer FP1 is extended by copying strand X' as a template and simultaneously

displaces the remainder of strand X. Similarly the other FP1 primer is extended by copying of strand X and displaces the remainder of strand X'. Simultaneously with extension of primers FP1, the 3' ends of the cleaved target molecule are extended using the FP1 primers as a template regenerating the restriction endonuclease sites. All of these copying reactions are schematically illustrated between steps (c1) and (c2) of Fig. 3.

For the purpose of simplicity, Fig. 3 shows the product of the strand displacement polymerisation (i.e. the product of step (c2)) to be two, foreshortened, double stranded copies of the original molecule (YX/X'Y' and XY'/YX'). It can be seen that each of these copies lacks one terminus of the original including the TspRI site at the end of the molecule.

As depicted, the next steps (steps (d) and (e)) involve cleavage of the molecules with *Tsp*RI and hybridisation of primers FP1 to the 3' overhangs produced. Once these primers are hybridised to the respective strands, strand displacing primer extension can occur as shown (steps (f1) and (f2)). The products of this extension include two foreshortened double stranded molecules (YX/X'Y' and XY'/YX') essentially identical to the products of steps (c1) and (c2). These molecules are, therefore, available for further cycles of TspRI cleavage/hybridisation/extension (steps (d), (e) and (f1/f2)). The other products of step f ((f1) and (f2)) are two, further shortened, single strand sequences (X and X') which contain neither TspRI restriction site. These sequences are complementary, being copies of each strand of the central region of the original molecule.

With the method as thus far described, these single strands X and X' do not participate further in the amplification reaction. Amplification may however be greatly enhanced by incorporating, in the reaction mix, additional primers ISO1 and ISO2 (see Fig. 3(g)). ISO1 has a 5' terminal sequence corresponding to Y above whereas the remainder of the primer may be hybridised to the 3' end of sequence X'

(Fig 3 (g)). Primer ISO2 also has 5' sequence corresponding to Y and the remainder of the primer may be hybridised to the 3' end of sequence X (Fig. 3 (g)). Polymerase extension, once again, "fills in" the molecules (steps (h1) and (h2)) to generate two foreshortened double stranded products. These molecules (YX/X'Y' and XY'/YX') are identical, in sequence terms, to the double stranded products of steps (c1/c2) and (f1/f2) described earlier, and so, are also able to re-enter further cycles of TspRI cleavage/hybridisation/extension (steps (d), (e) and (f1/f2)) described earlier.

Therefore, to summarise, each original molecule 1 undergoes a reaction resulting in the production of two shortened products lacking one or other terminal restriction site. Further processing of these molecules leads to their cyclical regeneration with the additional production of single stranded copies of each strand of the region between the *Tsp*RI sites in the original molecule. In the presence of the longer, target sequence specific ISO-primers (ISO1 and ISO2) these single strands are processed to generate further molecules identical to the earlier shortened double strands. The overall process is depicted in Fig. 3. The reaction thus cycles from (d) to (h) in a geometric manner.

It should be noted that primers ISO1 and ISO2 should be at much lower concentration than primer FP1 so that in step (b) of Fig. 3 there is a much higher probability of primer FP1 hybridising to the 3'-overhang than either of ISO1 or ISO2 which could not then be extended to re-generate the restriction site.

For the purposes of simplicity, the description so far has assumed that the reaction occurs in "discrete" steps. Thus for example, with reference to steps (c1) and (c2) it has been assumed that the copying reactions illustrated therein are completed before any further cutting of the molecule. In practice, this may not actually be the case since once the restriction site has been re-created it may be nicked once again before the copying reaction illustrated in steps (c1) and (c2) is completed. This has not however effected the overall result of the process as described above.

A number of modifications may be made to the process.

For example, the process may be performed using two different sequences FP1 and FP2 instead of the single sequence FP1 as described.

Furthermore, under certain conditions, the cleavage site (generated by *Tsp*RI) may not dissociate prior to extension by nucleotide polymerase so that, under such conditions, the amplification reaction will proceed without the need to use primer FP1. This is illustrated in the procedure of Fig. 4 which, in many respects, is analogous to the procedure shown in Fig. 3, save that:

(i) the primers FP1 are not used; and

(ii) the cutting of double stranded molecules is shown to yield seventeen base pair fragments designated SF1, which do not dissociate from the molecule and which, as will be appreciated from Fig. 4, have a priming function in the same manner as primers FP1.

In further modifications of the procedure, alternatively or additionally there may be only one restriction site per molecule or different restriction sites at the opposite ends of the molecule.

Alternatively or additionally the restriction site may be that of a restriction enzyme other than *Tsp*RI.

It will be appreciated that, if the sequence to be amplified is present in a single stranded molecule then a double stranded molecule containing the required restriction site may be generated therefrom using known techniques.

The invention is illustrated by the following non-limiting Examples.

Example 1

An amplification reaction was effected using the following templates, primers and reaction conditions.

Template 400 bp PCR fragment (Isofragment) generated from pUC19 using primers ISOS1 and ISOS2 below;

Primers: Two sequence-specific primers ISOS1 and ISOS2, and one Forcing Primer, ISOFP1 (equivalent to FP1 in Fig. 3) as shown below;

ISOS1 (SEQ ID NO. 1): 5'-TAATC TTTGG CAGTG GCTTA CAACG TCGTG ACTGG GAAAA C

ISOS2 (SEQ ID NO. 2): 5'-TAATC TTTGG CAGTG GCTGA CGGTG AAAAC CTCTG ACAC T

ISOFP1 (SEQ ID NO. 3): 5'-TAATC TTTGG CAGTG GC

Reaction Conditions:

20μL New England Biolabs Buffer 4
10μL Isofragment (50fmol μL^{-1})
5μ dNTPs (10mM each)
1μL ISOS2 (4 pmol μL^{-1})
2μL ISOS1 (2.2 pmol μL^{-1})
1μl ISOFP1 (40 pmol μL^{-1})
5μ*Tsp*RI (5 U μL^{-1})
1μL Bovine Serum Albumen (NEB 100x concentrated)

170 μL H_2O

A mix containing the three primers (ISOS1 and ISOS2 at approx. 20 fmol μL^{-1}, and ISOFP1 at approx. 200 fmol μL^{-1}), deoxyribonucleotides (250 $\mu molL^{-1}$ each), and template (Isofragment 2 at 2.5 fmol μL^{-1}) was equilibrated (in a water bath) to 65°C when 25 U *Tsp*RI restriction enzyme was added. The optimum temperature for cleavage of Isofragment 2 by *Tsp*RI is 65°C and incubation was continued for 20 minutes to allow restriction to occur (Fig. 3(a)). The mix was transferred to a second water bath at 37°C. A short equilibration period (30s - 1m) was then included to allow hybridisation of primers to exposed ssDNA ends of the fragment (Fig. 3(b)). 10U of Klenow (exo-) DNA polymerase was added, to extend the hybridisation products, which had accumulated, following restriction. The extension reaction was continued for 5 m at 37°C (Fig. 3(cl/c2)). The mix was transferred to the 65°C water bath to initiate a second restriction cleavage by *Tsp*RI (Fig. 3(d)). Following 20 minutes digestion and 30s - 1min hybridisation (Fig. 3(e) and (g)) further Klenow enzyme (10U) was added and extension continued for 5 minutes (Fig 3. (fl/2) and (h1/2)). Since *Tsp*RI can survive incubation at lower temperatures (e.g. 37°C) the initial amount input was sufficient for the entire reaction. In contrast, Klenow (exo-) is destroyed by prolonged incubation at 65°C and so additional enzyme was added to the mix following each incubation at the higher temperature. The cycle of incubation at 65°C, hybridisation, Klenow addition and extension at 37°C constitute the "cycles" of amplification. Samples (20 μL) were removed at the end of the cycle as necessary. The reaction in the samples taken was terminated immediately by the addition of 4μL of agarose gel loading dye and samples were held at 4°C until all samples were collected. The samples were then analysed by agarose gel electrophoresis in 1% (w/v) agarose gel, containing 0.5μg mL^{-1} ethidium bromide, in 1 x TBE buffer.

The results of this procedure are shown in Fig. 5 which demonstrates that amplification was clearly visible in all cases.

The above procedure was repeated but using an extension temperature of 39°C instead of 37°C. A repeat was also conducted using the extension temperature of 39°C but omitting the ISOFP1 primer from the reaction mix. The results of these procedures are shown in Fig. 6. The importance of including the primer ISOFP1 was clearly demonstrated by comparing amplification, at 39°C, in the presence and absence of the primer.

The effect of ISOFP1 on amplification can be quantified, to an extent, by using the gel scanning software (GELSCAN-UVP Ltd). To estimate peak heights or peak areas for the specific band being amplified (ca. 400 pairs). The results of peak area analysis for the gel photograph of Fig. 6 are illustrated in Fig. 7. These results show that there is considerable amplification of the fragment in the presence of ISOFP1. There is also a small, but significant, amplification in the absence of ISOFP1.

Example 2

An amplification reaction was effected using the following template, primers and reaction conditions.

Template

Purified ISOCMV template (144bp) at a concentration of 829fmol/µl (produced by PCR of Cytomegalovirus using ISOCMV001 and ISOCMV002 primers).

CMV FRAGMENT (SEQ ID NO. 4)

5' - TTAAGTTACG CACTGAGGAA TGTCAGCTTC CCAGCCTCAA GATCTTCATC
GCCGGGAACT CGGCCTACGA GTACGTGGAC TACCTCTTCA AACGCATGAT
TGACCTCAGC AGTATCTCCA CCGTCGAGAC AGTGGTGACA AGAC-3'

Primers

ISOCMV001 36-mer (SEQ ID NO. 5)
5' TTA AGT TAC GCA CTG AGG AAT GTC AGC TTC CCA GCC 3'

ISOCMV002 37-mer (SEQ ID NO. 6).
5' GTC TTG TCA CCA CTG TCT CGA CGG TGG AGA TAC TGC T 3'

FPCMV003 17-mer (SEQ ID NO. 7).
5' TTA AGT TAC GCA CTG AG 3'

FPCMV004 17-mer (SEQ ID NO. 8).
5' GTC TTG TCA CCA CTG TC 3'

FP primers were used at a concentration of 50pmol/µl while the longer ISO primers were used at a concentration of 5pmol/µl.

Reaction Conditions:

The following reactions were set up in 0.2ml flat-topped thin-walled PCR tubes and incubated, following enzyme addition, at 65°C (in a Biometra TRIO Thermoblock). The time points for incubation were 0, 20 40 and 80 minutes. At the appropriate time, 6µl of agarose gel loading dye was added to each reaction mix and samples were placed at 4°C until all time points were completed.

ISOCMV001 primer	2µl
ISOCMV002 primer	2µl
FPCMV003 primer	2µl
FPCMV004 primer	2µl
ISOCMV Template	0.5µl
50mM magnesium chloride (GIBCO)	6µl
10 x Thermopolymerase buffer (New England Biolabs)	3µl
10mM dNTP (Pharmacia)	1µl

*Tsp*RI restriction enzyme, 5U/µl (New England Biolabs)	2µl
Bst Polymerase, 4U/µl (New England Biolabs)	0.5µl
100 x Bovine Serum Albumin (New England Biolabs)	
(diluted to 1X with Molecular Biology grade water)	9µl
Total Reaction Volume	**30µl**

Following incubation twenty microlitres of each sample was analysed by loading on a 10% polyacrylamide, 1xTBE (non-denaturing) gel using a Bio-Rad Mini-PROTEAN gel system. The gel was run for approximately 1 hour at 100-150V and then stained for 20-30 minutes in a solution of 0.5 mg/ml ethidium bromide/1 x TBE (diluted from a stock of 10mg/ml solution from Sigma).

The gel was visualised using a UV transilluminator and photographic system (MWG Biotech). An image of the gel is show in Fig. 8 and demonstrates considerable amplification under the conditions used.

Example 3

Template: 400 bp PCR fragment (Isofragment) generated from pUC19 using primers ISOS1 and ISOS2 below;

Primers: Two sequence-specific primers ISOS1 and ISOS2, and one Forcing Primer, ISOFP1, as shown below;

ISOS1 (SEQ ID NO. 1): 5'-
TAATCTTTGGCAGTGGCTTACAACGTCGTGACTGGGAAAAC

ISOS2 (SEQ ID NO. 2): 5'-
TAATCTTTGGCAGTGGCTGACGGTGAAAACCTCTGACACAT

ISOFP1 (SEQ ID NO. 3): 5'- TAATCTTTGGCAGTGGC

Reaction Conditions:

20μL New England Biolabs Buffer 4
10μL Isofragment (50fmol μL^{-1})
5μL dNTPs (10mM each)
1μL ISOS2 (4pmol μL^{-1})
2μL ISOS1 (2.2 pmol μL^{-1})
1μL ISOFP1 (40pmol μL^{-1})
5μL *Tsp*RI (5 U μL^{-1})
1 μL Bovine Serum Albumen (NEB 100x concentrated)
170 μL H_20

A mix containing the three primers (ISOS1 and ISOS2 at approx. 20fmol μL^{-1}, and ISOFP1 at approx. 200 fmol μL^{-1}), deoxynucleotides (250 μmol μL^{-1} each), and template (Isofragment 2 at 2.5 fmol μL^{-1}) was equilibrated (in a water bath) to 65°C, when 25 U of *Tsp*RI restriction enzyme was added. Incubation was continued for 20 minutes when the mix was transferred to a second water bath at 34°C. After a short equilibration period (30s - 1 m) 10U of Klenow (exo-) DNA polymerase was added and extension continued for 5 m at 34°C prior to returning the mix to the 65°C water bath to initiate another 'cycle' of amplification. Samples (20 μL) were removed at the end of the cycle as necessary. The reaction in samples taken was terminated immediately by the addition of 4 μL of agarose loading dye and samples were held at 4°C until all samples were collected. The samples were then analysed by agarose gel electrophoresis in 1% (w/v) agarose gel, containing 0.5μg mL^{-1} ethidium bromide, in 1 x TBE buffer.

The results of this amplification procedure are shown in the gel photograph of Fig. 9 from which amplification is clearly demonstrated.

Fig. 10 is a plot showing the accumulation of product (400bp band) based on peak area analysis of the GelBase/GelBlot analysis package (Ultra Violet Products Limited, Cambridge, UK). The 10 cycle value is not shown.

These results show that there was considerable amplification of the fragment.

Example 4

Template: purified 144bp PCR fragment (CMV144) generated from human cytomegalovirus DNA using ISOCMV001 and ISOCMV002-AT primers below (used at a final concentration of 760 fmol/μl).

Primers:

ISOCMV001 (36-mer) (SEQ ID NO. 5):

5' TTA AGT TAC GCA CTG AGG AAT GTC AGC TTC CCA GCC 3'

ISOCMV002-AT (37-mer) (SEQ ID NO. 9):

5' ATA TTG TAA CCA CTG TCT CGA CGG TGG AGA TAC TGC T 3'

Both primers were used at a concentration of 5pmol/μl

Reagents:

Wheat-germ tRNA @ 10μg/ml (SIGMA) Lot 87H4045. Stock at 10mg/ml

50mM magnesium acetate (SIGMA) Lot No. 77H10581

10 x Thermopolymerase buffer (New England Biolabs) Lot No. 20

10mM dNTP (Pharmacia, Polymerisation mix) Lot 7122094021

TspRI restriction enzyme, 5U/μl (New England Biolabs) Lot No. 2

Bst Polymerase, 8U/μl (New England Biolabs) Lot No. 13B

Agarose Gel Loading dye, AGL007

Molecular Biology grade water from ELGA water purifier

Equipment:

Biometra TRIO Thermoblock

Method:

Template (CMV144) was diluted from the 760 fmol/µl stock using serial tenfold dilutions. Concentrations of template used in the assay were 760 zmol/µl (456000 molecules/µl), 76 zmol/µl (45600 molecules/µl), 7.6 zmol/µl (4560 molecules/µl), 0.76 zmol/µl (456 molecules/µl), and 0.076 zmol/µl (456 molecules/µl).

The following reaction was set up in 0.2ml flat-topped thin-walled PCR tubes, using each of the five template concentrations detailed above, and incubated at 56°C for 2 hours.

ISOCMV001 primer	2µl
ISOCMV002 primer	2µl
50mM MgAc	5µl
10mM dNTP	0.5µl
10 x Thermo buffer	5µl
TspRI	1.5µl
Bst Polymerase	0.5µl
template (CMV144) dilutions	0.5µl
MB water up to 25µl	7.5µl
Total Reaction Volume	**25µl**

Fifteen microlitres of sample was analysed by loading on a 10% polyacrylamide, 1xTBE (non-denaturing) gel using a Bio-Rad Mini-PROTEAN gel system. The gel was run for approximately 45 mins at 175V and then stained for 10 minutes in a solution of 0.5mg/ml ethidium bromide/1 x TBE (diluted from a stock of 10mg/ml solution from Sigma).

The gel was visualised using a UV transiluminator and photographic system (MWG Biotech). The results of this amplification procedure are shown in the gel photograph of Figure 11 from which amplification is clearly demonstrated.

In the above Examples, the targets used contained sites for the restriction enzyme, *Tsp*RI, which were introduced during their production. It will however be appreciated that suitable targets may be generated in a number of ways. Such methods might include, but are not limited to:

1. Restriction digestion of long nucleic acid sequences carrying the target, to generate fragments with at least one known end. The restriction enzyme may, or may not, be that used for subsequent amplification, however, for exponential amplification the ISO primers must contain 3' sequences homologous to the 3' sequences of the fragment produced. Denaturation of the fragment (e.g. by elevated temperature) similar in structure to X and X' shown in Fig. 3. (F2) and thus the amplification would begin from that point.

2. Ligation of two synthetic oligonucleotides, using the target molecule as template, in a manner that such ligation generates a product which, when separated from the target by denaturation, as above, has a structure YX or YX' or YXY' or YX'Y', using the nomenclature as in Fig. 3. Such molecules would be a target for hybridisation by FP1 or an appropriate ISO primer, and would generate double stranded intermediates in the amplification cycle following polymerase extension.

3. Reverse transcription of an RNA target molecule with a primer containing the target specific sequence flanked by a sequence containing a suitably positioned restriction endonuclease site (ISO primer). Following separation of the RNA from the extended DNA copy product the addition of a target specific "downstream" primer (or ISO primer) would initiate the amplification reaction.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: TEPNEL MEDICAL LIMITED
(B) STREET: UNIT 8, ST. GEORGE'S COURT, HANOVER BUSINESS PARK
(C) CITY: ALTRINCHAM
(D) STATE: CHESHIRE
(E) COUNTRY: UNITED KINGDOM
(F) POSTAL CODE (ZIP): WA14 5UA

(ii) TITLE OF INVENTION: AMPLIFICATION OF NUCLEIC ACIDS

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TAATCTTTGG CAGTGGCTTA CAACGTCGTG ACTGGGAAAA C 41

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TAATCTTTGG CAGTGGCTGA CGGTGAAAAC CTCTGACACT 40

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TAATCTTTGG CAGTGGC 17

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTAAGTTACG CACTGAGGAA TGTCAGCTTC CCAGCCTCAA GATCTTCATC GCCGGGAACT 60

CGGCCTACGA GTACGTGGAC TACCTCTTCA AACGCATGAT TGACCTCAGC AGTATCTCCA 120

CCGTCGAGAC AGTGGTGACA AGAC 144

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTAAGTTACG CACTGAGGAA TGTCAGCTTC CCAGCC 36

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GTCTTGTCAC CACTGTCTCG ACGGTGGAGA TACTGCT 37

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TTAAGTTACG CACTGAG 17

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTCTTGTCAC CACTGTC 17

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATATTGTAAC CACTGTCTCG ACGGTGGAGA TACTGCT 37

CLAIMS

1. A method of amplifying a nucleic acid sequence present in a first strand of a double stranded nucleic acid molecule comprised of complementary first and second strands wherein

said molecule incorporates an unmodified recognition site for a restriction enzyme capable of cutting the first strand at the 5' end of the sequence therein to be amplified and leaving the 3'-end region of the second strand projecting beyond the cut site in the first strand, and said method comprises

treating said nucleic acid molecule with said enzyme in the presence of a strand displacing polymerase and unmodified nucleotides for incorporation in an extending nucleic acid strand under conditions such that there is or becomes hybridised to said 3'-end region of the second strand a primer sequence complementary thereto whereby said primer sequence is extended in the 5' to 3' direction using the second strand as a template to re-generate the restriction endonuclease cut site and displace the sequence to be amplified.

2. A method as claimed in claim 1 wherein the nucleic acid molecule incorporates two of said restriction sites, one each side of the sequence to be amplified.

3. A method as claimed in claim 1 or 2 wherein the or each restriction site is a *Tsp*RI site.

4. A method as claimed in any one of claims 1 to 3 wherein the reaction mixture additional incorporates an excess of oligonucleotide primers (FP) incorporating the restriction sequence and capable of hybridising to the 3' end of the displaced sequence whereby a double stranded molecule incorporating the restriction site is generated and

the latter double stranded molecule is capable of participating in further cutting and extension/displacement reactions to generate strands which do not incorporate the restriction sequence.

5. A method as claimed in any one of claims 1 to 4 wherein the reaction mixture additionally incorporates oligonucleotide primers (ISOS) incorporating the restriction sequence and being capable of hybridising to said strands which do not incorporate the restriction sequence.

6. A method as claimed in any one of claims 1 to 5 wherein the nucleic acid is DNA.

7. A method as claimed in any one of claims 1 to 6 wherein the strand displacing polymerase is 9°N polymerase, Klenow (exo-) polymerase, Bst polymerase, Vent (exo-) polymerase, or Deep Vent (exo-) polymerase.

8. A method as claimed in any one of claims 1 to 7 effected isothermally.

9. A method as claimed in any one of claims 1 to 8 wherein the DNA molecule is generated in situ by reaction of a precursor nucleic acid molecule containing the sequence to be amplified with at least one primer capable of generating the restriction site.

5'--------NNCAGTGNN--------3'

3'--------NNGTCACNN-------5'

FIG.1

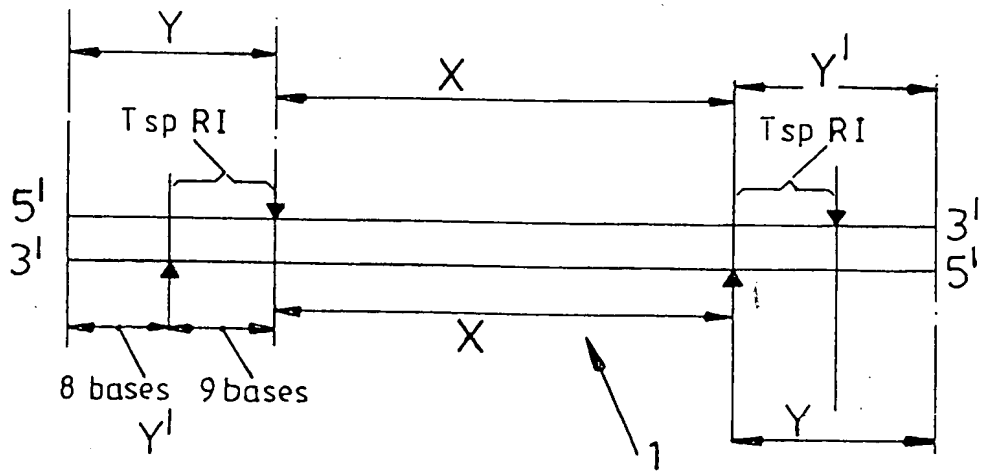


FIG.2

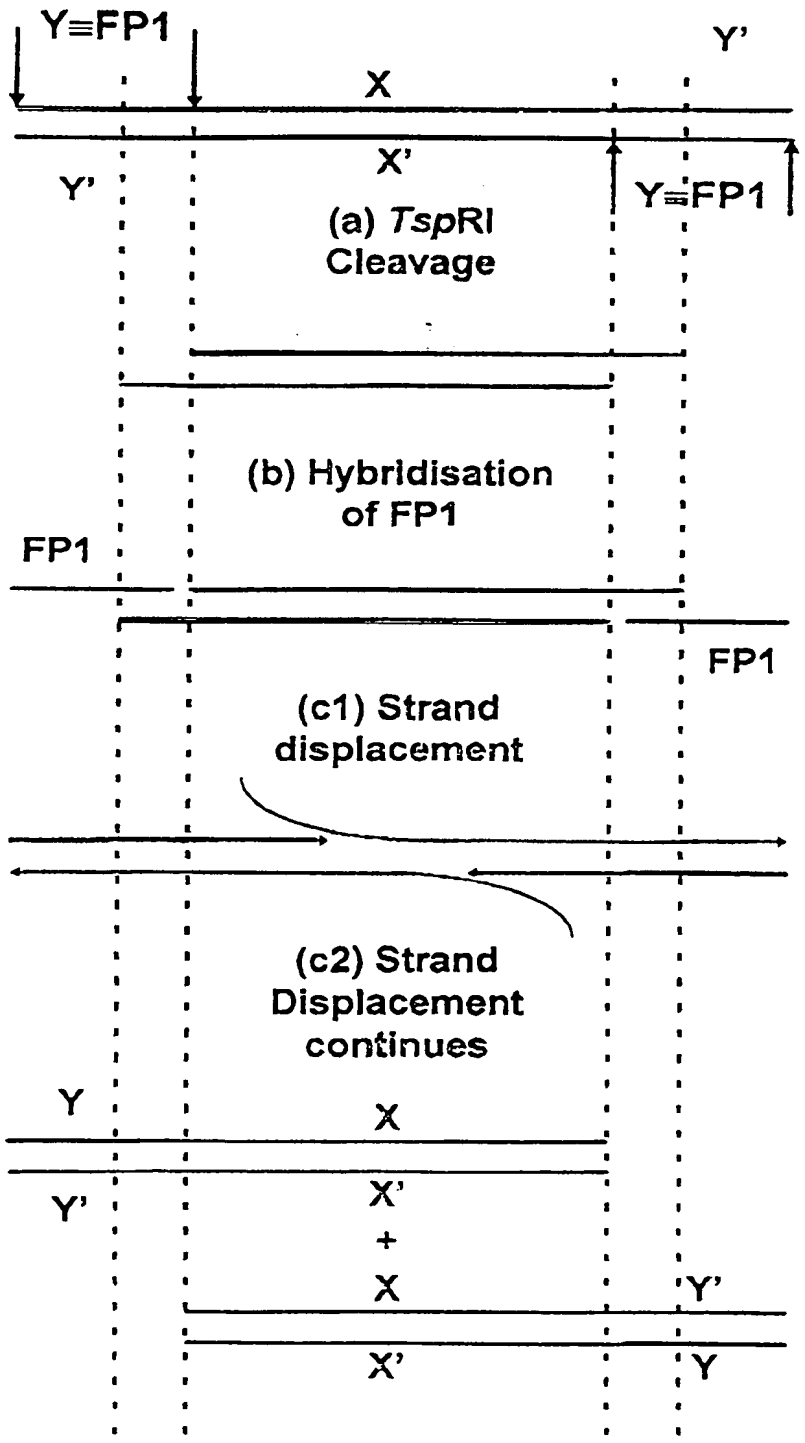

Y≡FP1
Y'
X
X'
Y'
Y≡FP1
(a) TspRI Cleavage
(b) Hybridisation of FP1
FP1
FP1
(c1) Strand displacement
(c2) Strand Displacement continues
Y
X
Y'
X'
+
X
Y'
X'
Y

FIG. 3/1

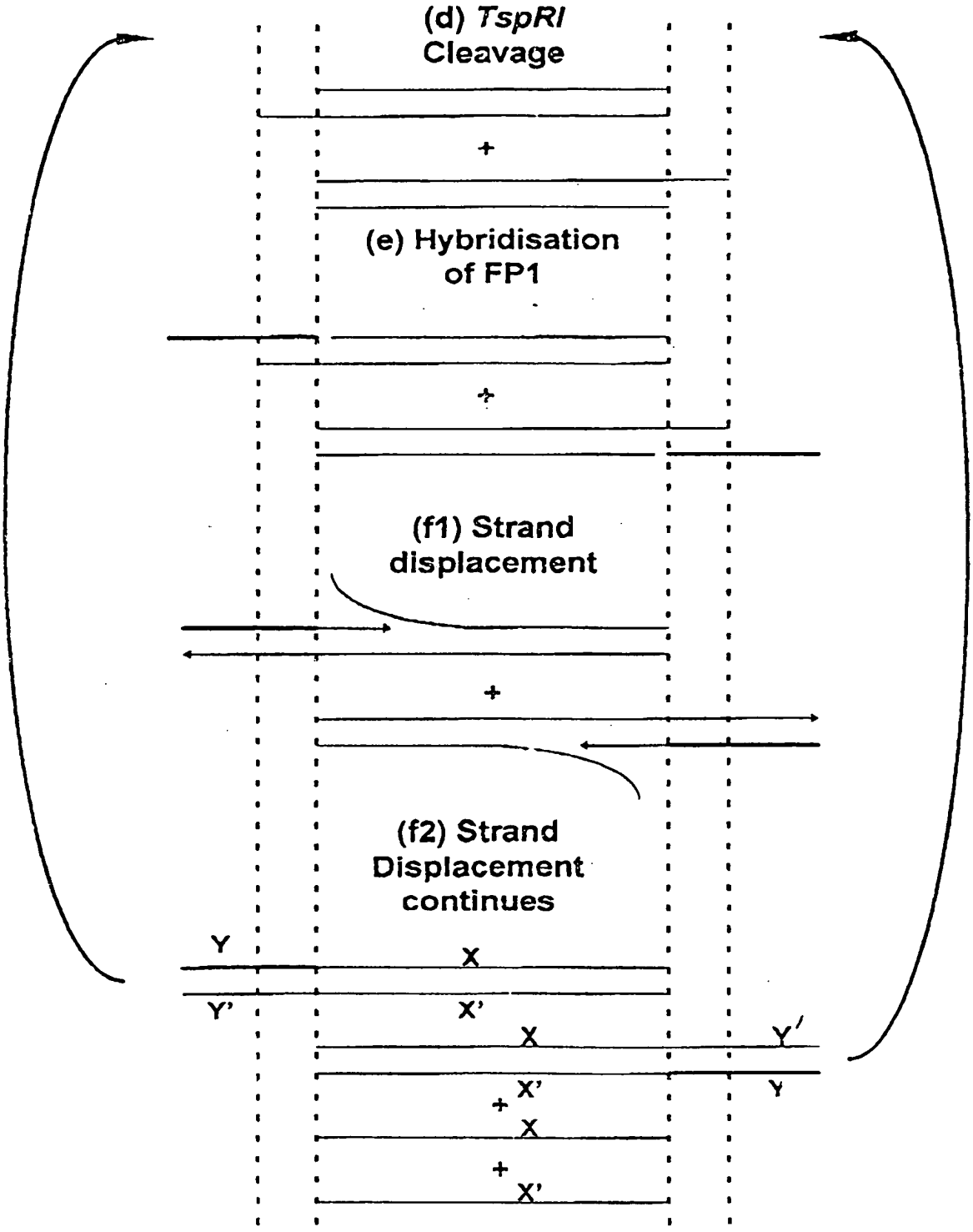

(d) TspRI Cleavage
+
(e) Hybridisation of FP1
+
(f1) Strand displacement
+
(f2) Strand Displacement continues
Y
X
Y'
X'
X
Y'
X'
Y
+
X
+
X'

FIG. 3/2

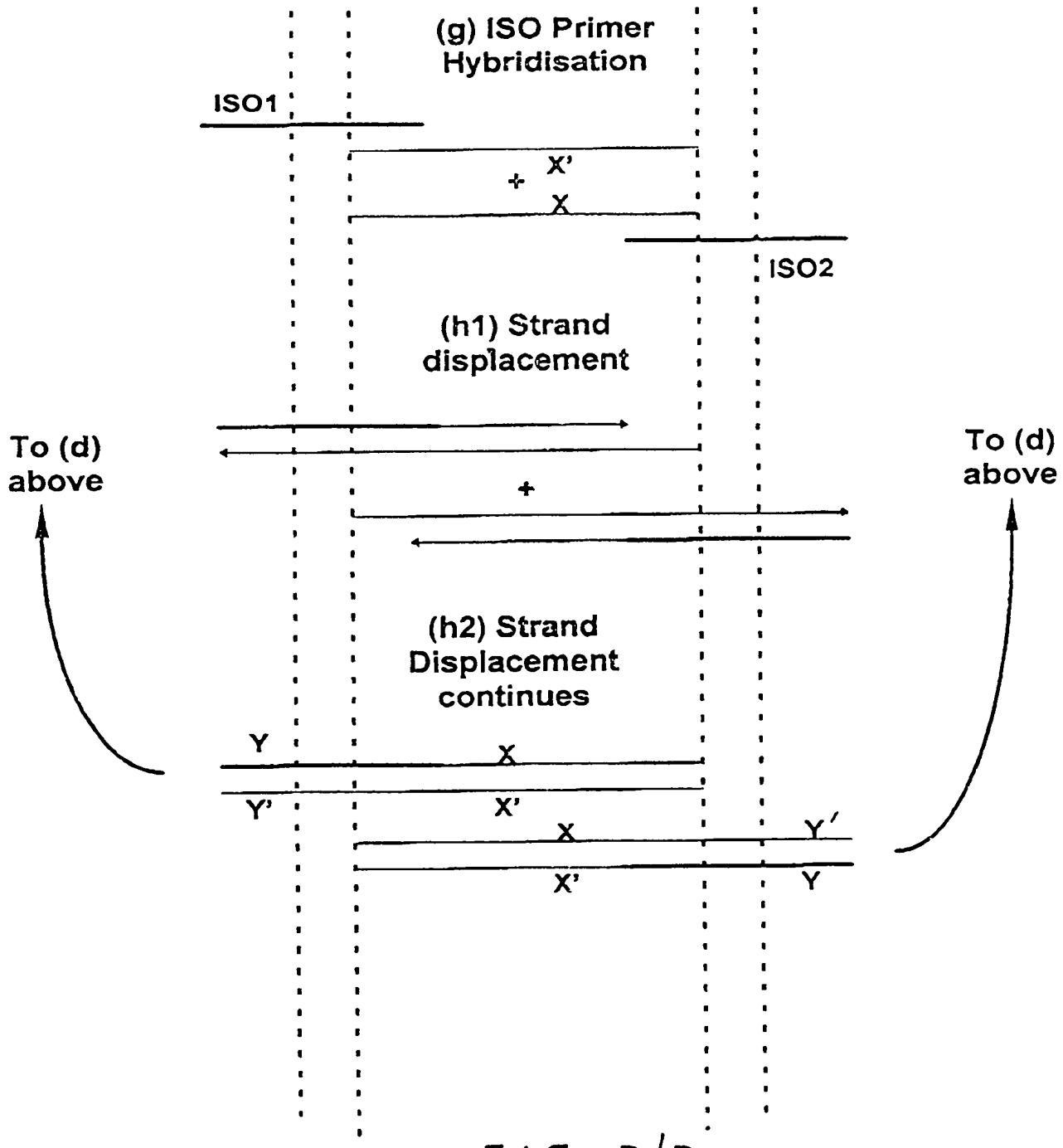

(g) ISO Primer Hybridisation
ISO1
X'
+
X
ISO2
(h1) Strand displacement
To (d) above
+
To (d) above
(h2) Strand Displacement continues
Y
X
Y'
X'
X
Y'
X'
Y

FIG. 3/3

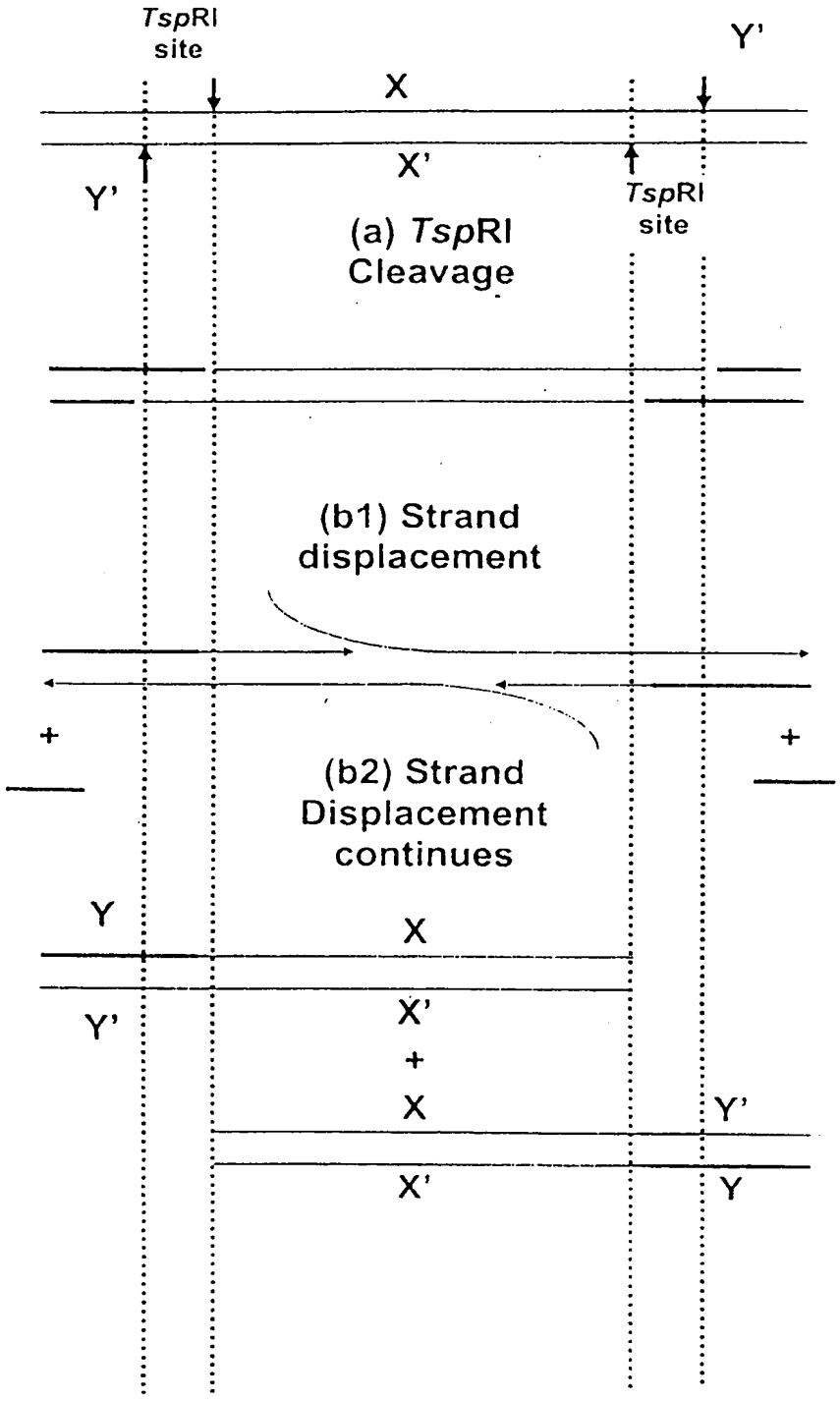

TspRI site
Y'
X
X'
Y'
TspRI site
(a) TspRI Cleavage
(b1) Strand displacement
+
+
(b2) Strand Displacement continues
Y
X
Y'
X'
+
X
Y'
X'
Y

FIG. 4/1

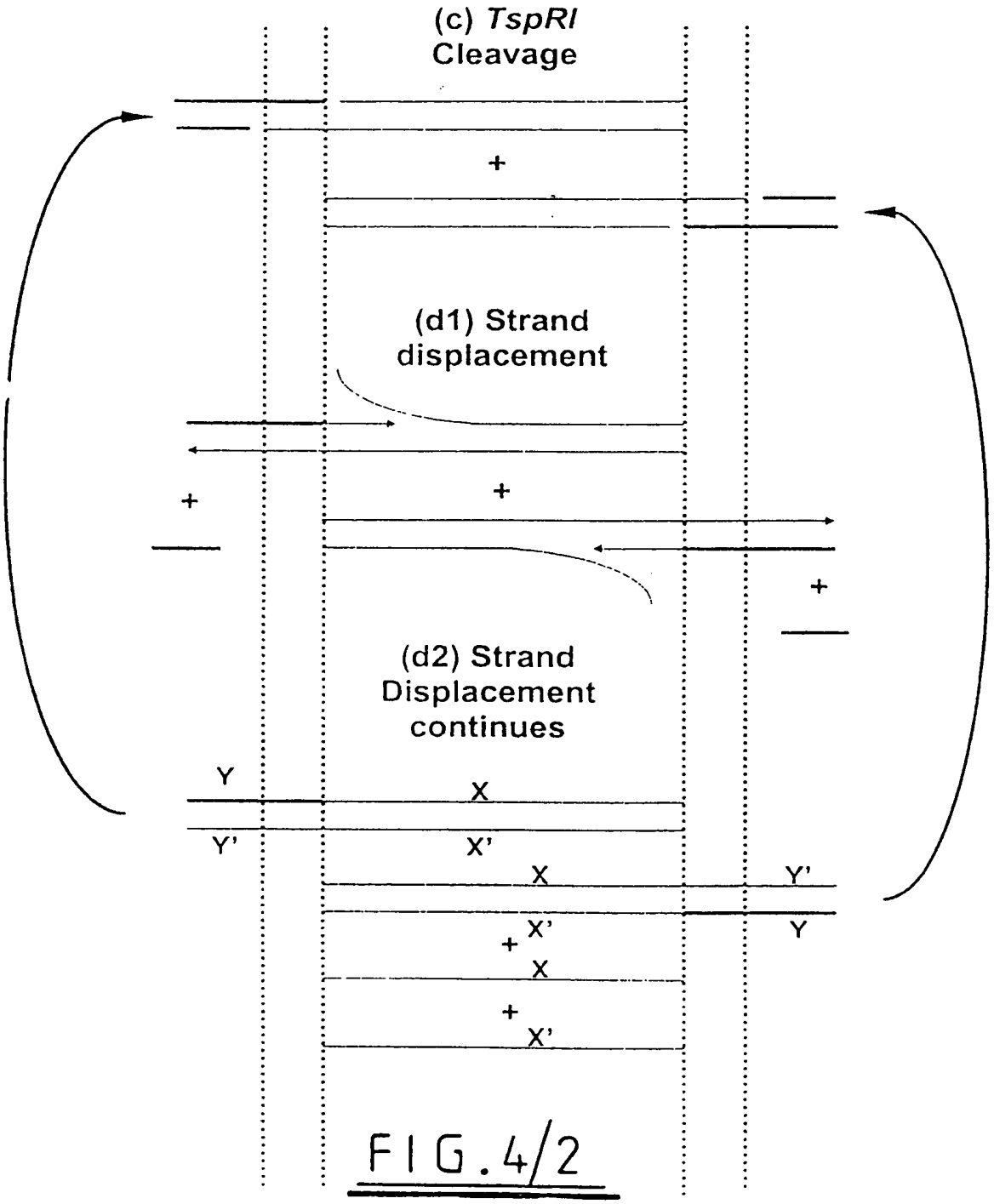

(c) TspRI Cleavage
+
(d1) Strand displacement
+
+
+
(d2) Strand Displacement continues
Y
X
Y'
X'
X
Y'
X'
Y
+
X
+
X'

FIG. 4/2

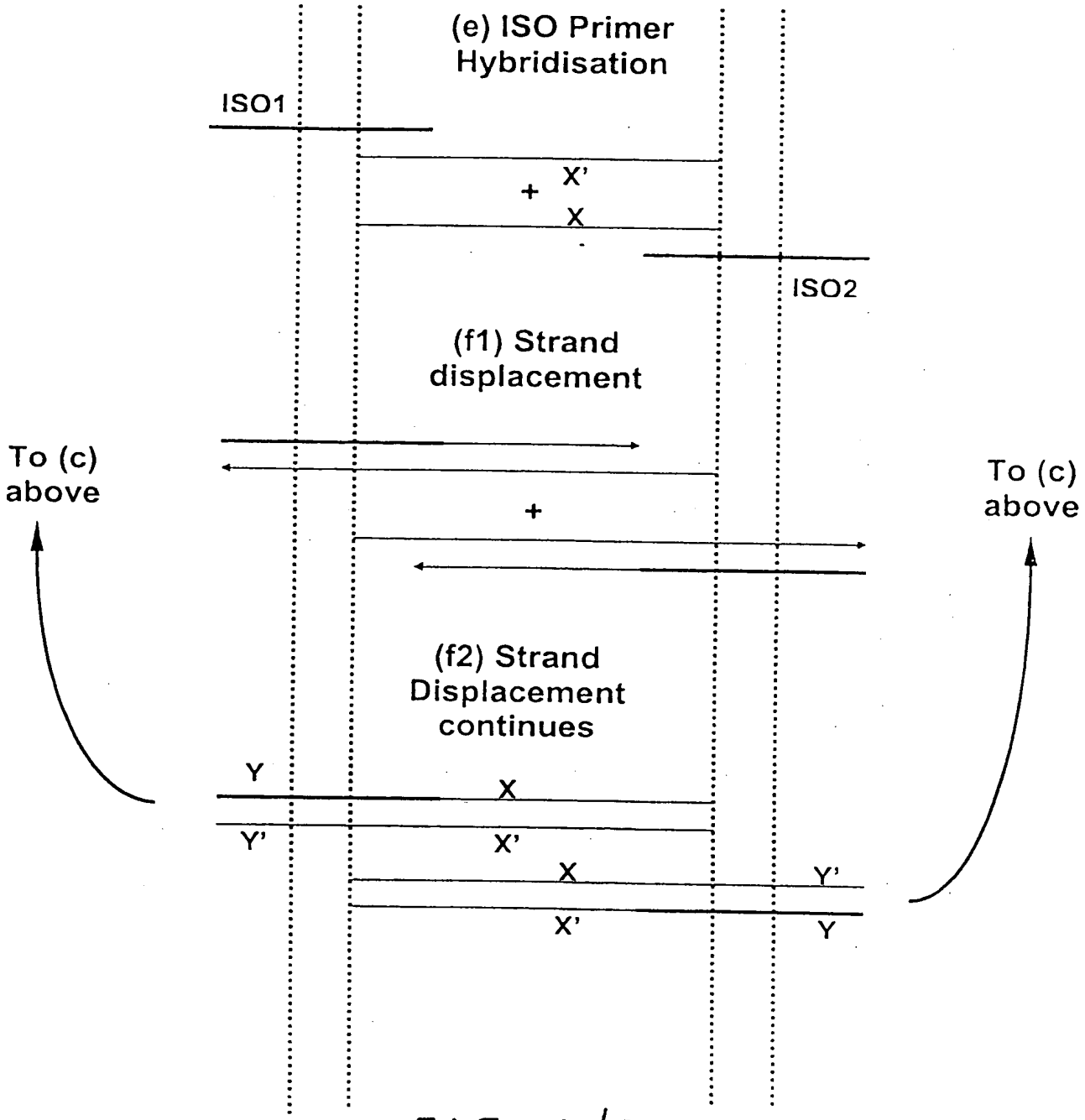
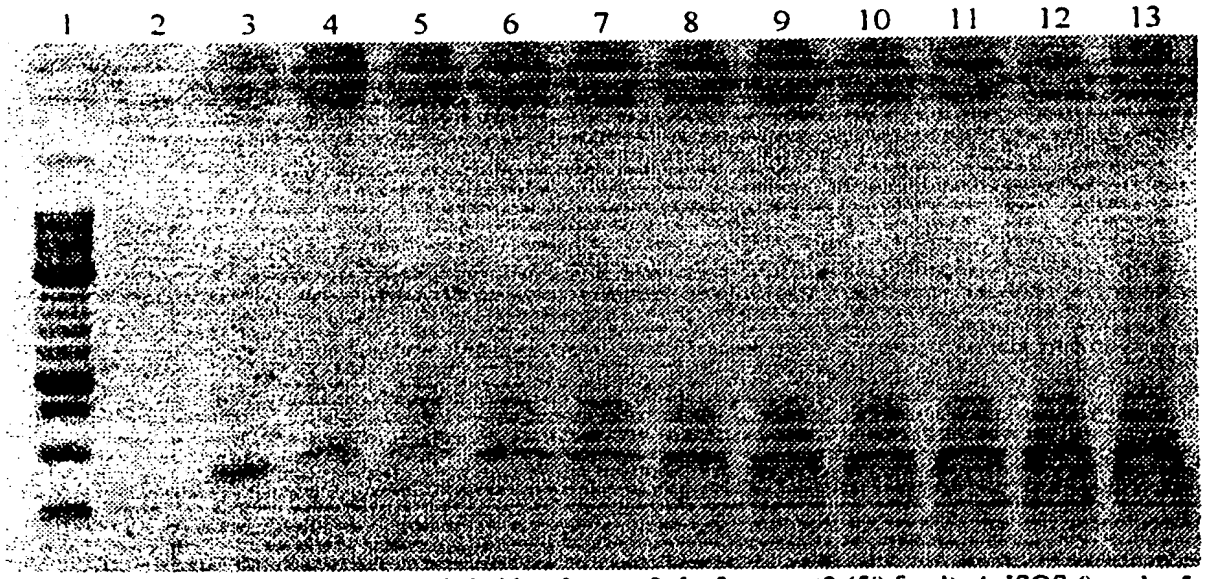

(e) ISO Primer Hybridisation
ISO1
X'
+
X
ISO2
(f1) Strand displacement
To (c) above
+
To (c) above
(f2) Strand Displacement continues
Y
X
Y'
X'
X
Y'
X'
Y

FIG. 4/3



(inverse image). Lane 1, 250 base pair ladder; 2, gap; 3, Isofragment2 (50 fmol); 4, ISOS-0 cycle; 5, ISOS -1 cycle; 6, ISOS -2 cycle; 7, ISOS -3 cycle; 8, ISOS -4 cycle; 9, ISOS -5 cycle; 10, ISOS -6 cycle, 11, ISOS -7 cycle; 12, ISOS -8 cycle; 13, ISOS -9 cycle.

Fig. 5

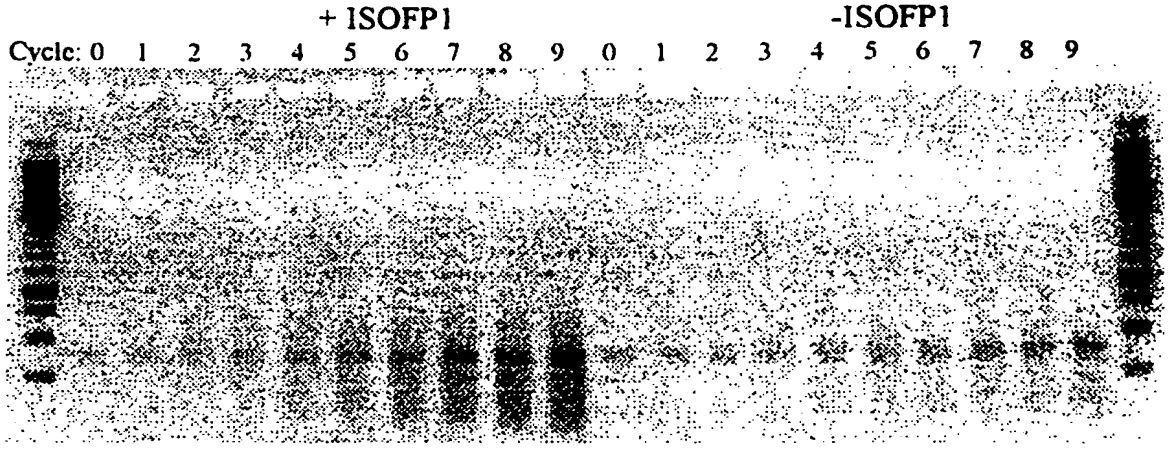


Fig. 6

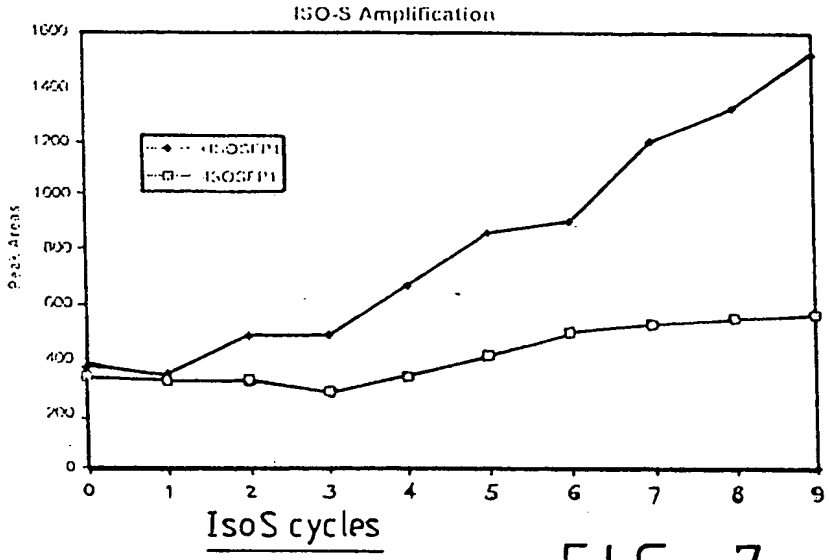

ISO-S Amplification
Peak Areas
0
200
400
600
800
1000
1200
1400
1600
0 1 2 3 4 5 6 7 8 9
IsoS cycles

FIG. 7

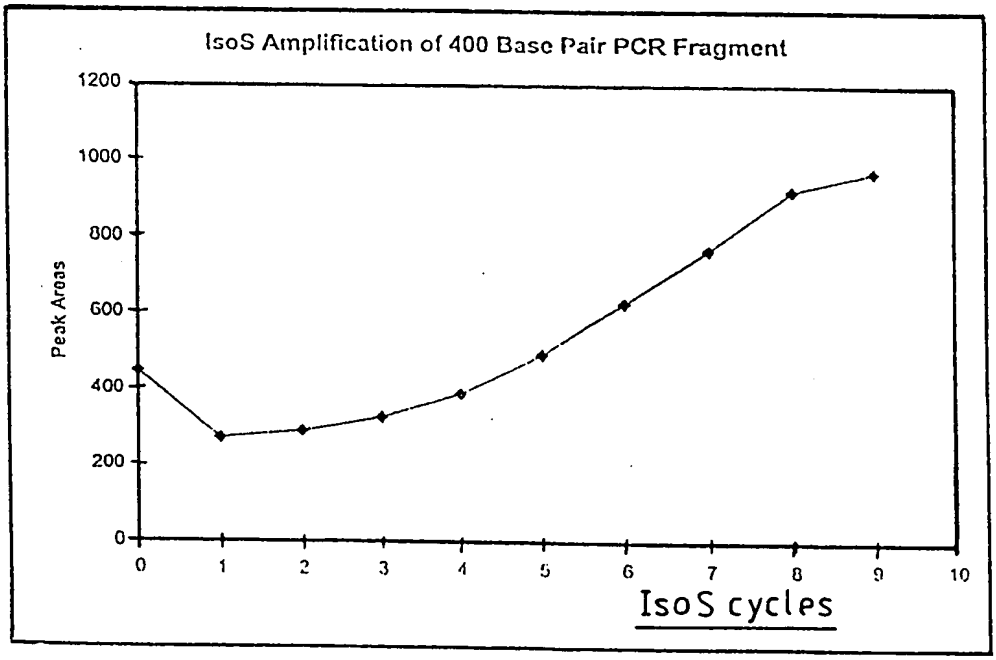
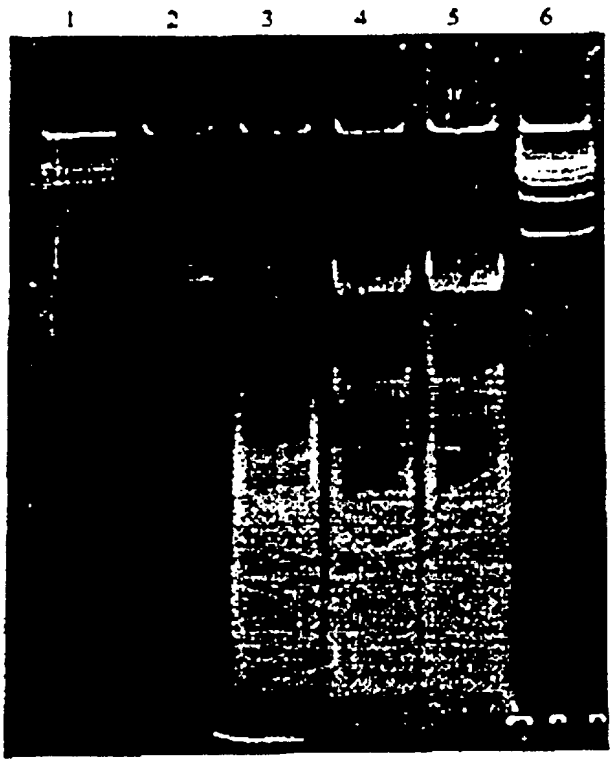
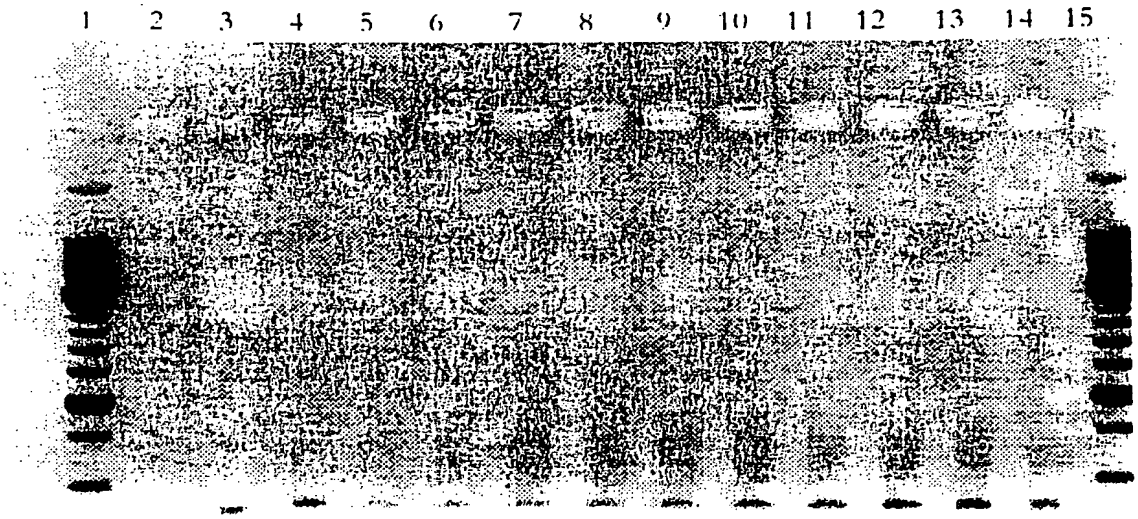

IsoS Amplification of 400 Base Pair PCR Fragment
Peak Areas
0
200
400
600
800
1000
1200
0 1 2 3 4 5 6 7 8 9 10
IsoS cycles

FIG. 10



Lanes 1 and 6; Standards (100bp ladder). Lane 2; Zero time. Lane 3; 20 minutes. Lane 4; 40 minutes. Lane 5; 80 minutes.

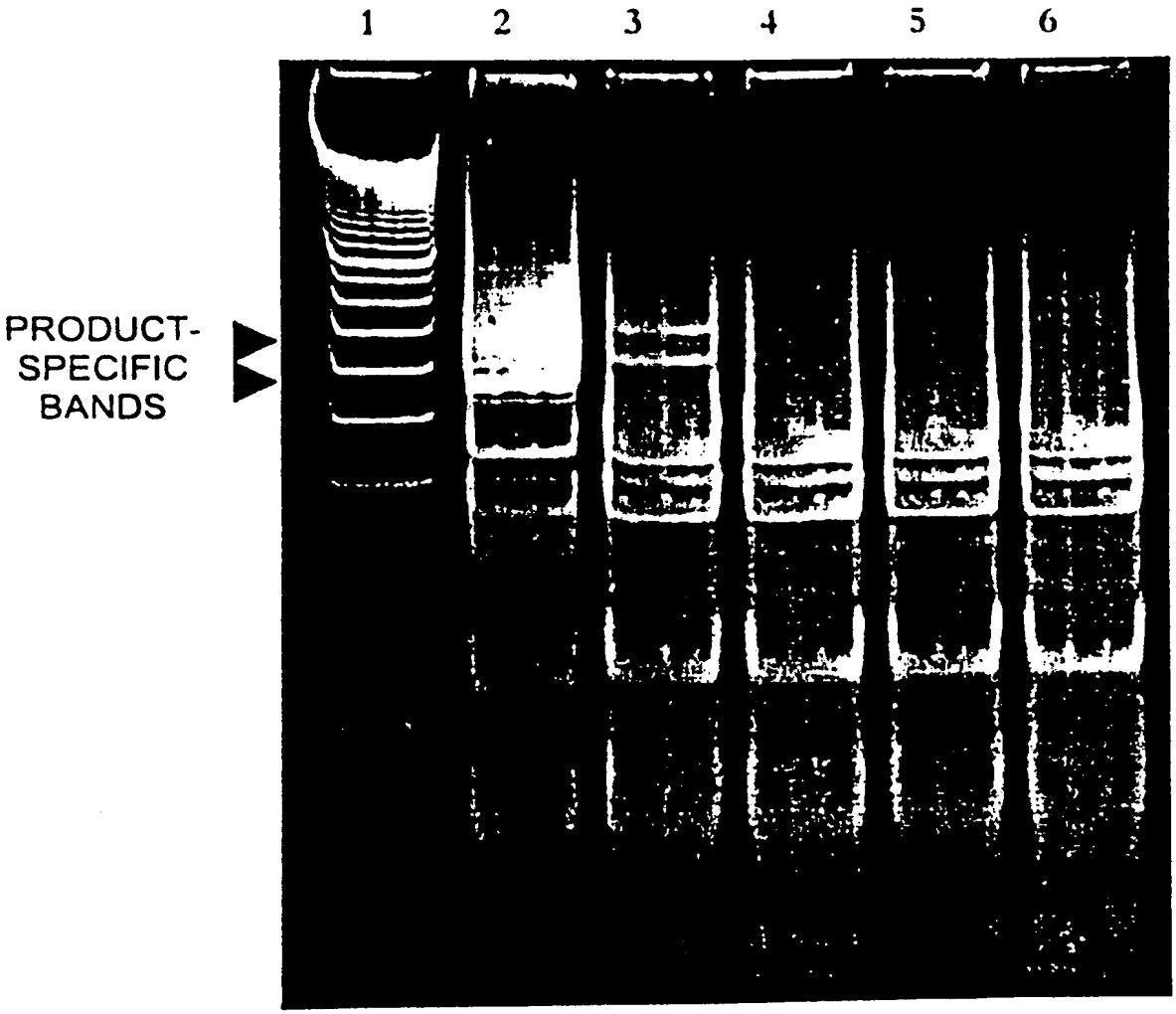
Fig. 8



(inverse image). Lanes 1 and 15, 250 base pair ladder; 2, gap; 3, Isofragment2 (50 fmol); 4, ISOS-0 cycle: 5, ISOS -1 cycle; 6, ISOS -2 cycle; 7, ISOS -3 cycle; 8, ISOS -4 cycle; 9, ISOS -5 cycle; 10, ISOS -6 cycle; 11, ISOS -7 cycle; 12, ISOS -8 cycle; 13, ISOS -9 cycle; 14, ISOS -10 cycle*.

*The 10 cycle sample was the remainder of the reaction mix and was less than the 20µL taken for other samples

Fig. 9



Lane 1: **20 bp DNA standard ladder**
Lane 2: 760 zmol template input
Lane 3: 76 zmol template input
Lane 4: 7.6 zmol template input
Lane 5: 0.76 zmol template input
Lane 6: Iso-S no template

Fig. 11

INTERNATIONAL SEARCH REPORT

Interi nal Application No
PCT/GB 98/02427

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FU D -J ET AL: "SEQUENCING DOUBLE-STRANDED DNA BY STRAND DISPLACEMENT" NUCLEIC ACIDS RESEARCH, vol. 25, no. 3, 1997, pages 677-679, XP000196977 see whole doc., esp. p. 679, 1. column ---	1-9
Y	WALKER G T ET AL: "STRAND DISPLACEMENT AMPLIFICATION - AN ISOTHERMAL, IN VITRO DNA AMPLIFICATION TECHNIQUE" NUCLEIC ACIDS RESEARCH, vol. 20, no. 7, 1992, pages 1691-1696, XP002019521 see whole doc, esp. figure 2 --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1998	23/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ınal Application No
PCT/GB 98/02427

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 25180 A (GEN PROBE INC) 21 September 1995 see claims -----	1-9

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr ıal Application No
PCT/GB 98/02427

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9525180 A	21-09-1995	AU 687535 B	26-02-1998
		AU 2101695 A	03-10-1995
		EP 0676476 A	11-10-1995
		JP 9510351 T	21-10-1997

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